University Boulevard, Manassas, VA 20110-2209 as suggested by the Examiner. Applicants respectfully request reconsideration and withdrawal of this objection.

(b) The use of trademarks in the instant specification has been objected to. Applicants have amended the specification to capitalize the recitations with the generic terminology in order to address the Examiner's concerns. Reconsideration and withdrawal of specification objection is respectfully requested.

Scope of Enablement

21) The Examiner has rejected the full scope of the claims as non-enabled since the specification allegedly lacks enablement or direction as to how to obtain modified pneumolysins that concomitantly have functions while carrying out the amino acid substitutions at positions 17, 18, 33, 41, 45, 46, 63, 83, 101, 102, 128, 189, 239, 243, 255, or 257. However, applicants respectfully traverse this ground for rejection.

Applicants assert that the specification enables modified pneumolysin polypeptides where at least one amino acid of SEQ ID NO:3 is substituted and the substitution occurs at a positions selected from the group consisting of positions: 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, where when the modified pneumolysin possesses only one substitution, the substitution is selected from the group consisting of positions: 61, 148, and 195, and the modified pneumolysins are soluble, elicit antibodies which are cross-reactive with wild-type pneumolysin, and have attenuated hemolytic activity. These functional and structural characteristics are identified by methods described in the instant invention and the skilled artisan would understand how to identify modified pneumolysin polypeptides from reading the instant specification. Specifically, the Examiner's attention is directed to page 23, ln. 20 through page 26, ln. 14, where modified pneumolysin polypeptides have reduced hemolytic activity (Tables 1-2). Modified pneumolysin polypeptides having the above-identified amino acid substitutions as described by the instant specification are "effective for reducing or eliminating the hemolytic activity...provided that at least one epitope capable of binding an antibody which binds native pneumolysin" (pg. 25, ln. 18 through pg. 27, ln. 2; Table 3) is maintained. Applicants

point out that the instant specification discloses methods for producing and identifying modified pneumolysins having reduced hemolytic activity by substituting amino acids (pgs 12-28) at various specific positions. Furthermore, because the claimed modified pneumolysins retain sufficient epitopes to be immunogenic and elicit antibodies which are cross-reactive with wild-type pneumolysin (pg. 11, lns. 18-22 and page 20, lns. 19-28), one skilled in the art would be capable from the description and guidance found in the instant specification to identify the claimed modified pneumolysin polypeptides having such properties.

Example 5 provides guidance as to how one skilled in the art would determine whether modified pneumolysins are soluble (Ex. 5, section (c) "testing for expression of modified pneumolysin polypeptides in the soluble fractions," pgs. 50-51). Testing the polypeptides for the preferred properties simply entails growing bacteria containing expression vectors encoding modified pneumolysins, inducing expression, lysing cells, and testing for characteristics such as hemolytic activity (where the polypeptide must first be solubilized), immunogenicity, and refoldability by circular dichrosim (CD) or fluorescence spectroscopy.

> It is well settled that patent applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art. *In re Angstadt, 537 F.2d 498, 502-03, 190 U.S.P.Q. (BNA) 214, 218 (CCPA 1976).* However, there must be sufficient disclosure, either through illustrative examples or terminology, to teach those of ordinary skill how to make and how to use the invention as broadly at it is claimed. (Emphasis added).
>
> *In re Vaeck*, 947 F.2d 488, 496, 20 USPQ 2D 1438 (Fed. Circ. 1991).

Applicants' specification provides sufficient direction to obtain pneumolysin mutants within the full scope of the claims and to determine whether they are hemolytically attenuated, soluble, and immunogenic. Based on applicants' disclosure, the ability to obtain and identify claimed pneumolysins is well within the state of the art or the relative skill of

those in the art. For example, the mutagenesis methods may be PCR-based and the hemolysis screening simply involves solubilizing the polypeptide, incubation and routine detection, all methods commonly known to those skilled in the art. Lastly, the breadth of applicants' claims is not overly-broad since the modified pneumolysins must comprise at least one amino acid substitution at a discrete location as described in claim 35. Therefore, applicants respectfully request reconsideration and withdrawal of this ground of rejection. No new matter has been introduced by this amendment. Reconsideration and withdrawal of this lack of enablement rejection is respectfully requested. (Additional discussion is found in paragraph 24 below).

35 U.S.C. §112, First Paragraph

22) Claims 35-37, 42, 53, and 60-79 have been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Applicants respectfully traverse this ground of rejection.

Specifically, the Examiner rejects claims 35-37, 42, 53, and 60-79 for the addition of a new limitation in the claim(s) that is considered to be new matter. The Examiner contends that the limitation of base claim 35, i.e. "refoldability" has been replaced by "partially-soluble," now amended to be - -soluble- - without providing support in the specification. However, applicants assert that the instant specification provides enabling description of methods for selecting modified pneumolysin polypeptides with the following preferred characteristics: "1) no hemolytic activity; 2) substantially full-length; 3) partially soluble, and 4) monomeric and refoldable" (Example 5). Since solubility is one of the preferred characteristics of a modified pneumolysin polypeptide of the invention, the instant specification provides support for replacing "refoldability" with - -soluble- -.

Briefly, Example 5 describes a screening method for modified pneumolysin polypeptides having the aforementioned characteristics. In particular, this section discusses methods for identifying modified pneumolysin polypeptides in soluble fractions, specifically in section (c) (pg. 50, ln. 30 – pg. 51, ln. 20) where "Modified pneumolysin polypeptides

expressed in both the soluble fraction and inclusion bodies are more likely to be refoldable." In order to identify modified pneumolysin polypeptides having reduced hemolytic acitvity, the pelleted lysates containing the polypeptides are solubilized with a denaturant. This procedure measures activity after the protein has undergone refolding (pg. 22, ln. 27 – pg. 23, ln. 4). If modified hemolytically attenuated pneumolysins are properly refoldable, then their hemolytic attenuation is more likely due to a functional mutation and not a structural mutation that causes the loss of native-antigenic structure. Since the characteristics of refolding and solubility are described and methods of testing for solubility and refoldability are provided in the instant specification, one skilled in the art would have sufficient guidance to identify modified pneumolysin polypeptides that possess the claimed characteristics, i.e., soluble, capable of eliciting antibodies that are cross-reactive to wild-type pneumolysin, and have attenuated hemolytic activity with one or more amino acid substitutions.

Applicants assert that the instant specification fully describes refoldable modified pneumolysins and procedures for assessing refoldability and solubility. Accordingly, these aspects of applicants' invention were clearly in applicants' possession as of the filing of the present application. The specification supports claims 35-37, 42, 53, and 60-79. Applicants respectfully request reconsideration and withdrawal of this §112, first paragraph rejection.

23) Claims 69-79 have been rejected under 35 U.S.C. §112, first paragraph for lack of descriptive support within the instant specification for polypeptides having various combinations of substitutions. Applicants respectfully disagree with the Examiner's grounds for rejection, but have amended the claims for clarification.

Applicants assert that the specification enables one skilled in the art as to how to identify without undue experimentation a pneumolysin mutant that is soluble and capable of eliciting antibodies that are cross-reactive with wild-type pneumolysin, with reduced hemolytic activity, having amino acid substitutions including a combination of substitutions at positions 17, 18, 61, 66, and 101 or positions 41, 172, 195, and 255 or positions 63, 127, 128, and 148 or positions 33, 46, 83, 239, and 257 or positions 45, 102, 189, and 195. Since a pneumolysin mutant having a single or any combination of the aforementioned amino acids

resulted in reduced hemolytic activity (page 51, lines 21 - page 52, ln. 20, Tables 4 & 5A), one skilled in the art would reasonably expect that a pneumolysin mutant having a combination of these same amino acid substitutions and additional amino acid substitutions would also possess reduced hemolytic activity. See for example Table 5A which reports clones having various claimed amino acid substitution combinations, (i.e., claims 69-79). Table 4 further reports the corresponding hemolytic activity which is well within the range indicating low hemolytic activity for each of the clones. Mutants pNVJ22 and pNVJ1 have 5 amino acid substitutions and less than 1 % hemolytic activity when compared to wild type (pNV19). Mutants pNVJ20, pNVJ45, and pNVJ56 have 4 amino acid substitutions and less than 1 % hemolytic activity when compared to wild type. Using these substitutions as guidance, the skilled artisan would be knowledgeable as to how to design other mutants without undue experimentation.

Applicants contend that the skilled artisan would be able to identify modified pneumolysin polypeptides that are soluble, capable of eliciting antibodies cross-reactive with wild-type pneumolysin, and have attenuated hemolytic activity, from those mutants having a combination of amino acid substitutions selected from the substitutions at positions 17, 18, 61, 66, and 101 or positions 41, 172, 195, and 255 or positions 63, 127, 128, and 148 or positions 33, 46, 83, 239, and 257 or positions 45, 102, 189, and 195. The quantity of experimentation is not undue because the experimentation is routine. The written description as previously described provides a reasonable amount of guidance as to how to obtain modified pneumolysin polypeptides having the characteristics of solubility, capability to elicit antibodies which are cross-reactive with wild-type pneumolysin, and reduced hemolytic activity.

The Examiner is reminded that the written description requirement can be met by disclosing identifying characteristics, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when related to a known or disclosed correlation between function and structure, or some combination of such characteristics for identifying the preferred modified pneumolysin polypeptides. (*PPG Indus., Inc. v. Guardian Indus. Corp.*, 75 F.3d 1558, 1564, 37 USPQ 2D 1618, 1623 (Fed. Cir. 1996); *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 296 F.3d 1316 (Fed. Cir. 2002)). The instant specification provides

such structural and functional properties for identifying the pneumolysin mutants. Furthermore, guidance is provided as to how the skilled artisan would test for hemolytic activity, solubility, antibodies which are cross-reactive with wild-type pneumolysin, and refoldability in order to identify modified pneumolysin polypeptides of the invention.

The specification as originally filed provides support, enablement, and a reasonable amount of guidance for the limitation encompassing a polypeptide having any combination of substitutions at residues 17, 18, 61, 66, and 101 or 41, 172, 195, and 255 or 63, 127, 128, and 148 or 33, 46, 83, 239, and 257. Reconsideration and withdrawal of this §112, first paragraph rejection is respectfully requested.

24) The Examiner has rejected claims 35-37, 42, 53, and 60-79 under 35 U.S.C. §112, first paragraph for allegedly not providing enablement for a soluble and haemolytically attenuated modified pneumolysin having one or more amino acid substitutions at any of the recited positions. Applicants respectfully traverse this ground of rejection; however, claim 35 has been amended to clarify that at least one amino acid substitution is selected from position 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, where when the modified pneumolysin possesses only one substitution, the substitution is selected from positions 61, 148, and 195.

As an initial matter, applicants assert that the element of 'refoldability' is replaced with a different element, i.e., solubility. Since solubility is a probabilistic indicator of refoldability, applicants do not equate the meanings of 'refoldability' and 'soluble.' Applicants assert that amended claim 35 does not introduce new matter. Support may be found in the instant specification as previously discussed in paragraph 22.

The Examiner has specifically pointed to single mutant pneumolysin species, pNV103, pNV207, pNV111, and pNV211 (of Table 5A) which possess a specific amino acid substitution. The Examiner contends that there is no showing that these mutants are soluble and further contends that there is no enablement for other single mutants that are encompassed in the scope of the claims which have attenuated hemolytic activity and retain the function(s) of immunogenicity/antigenicity and the ability to bind with an antibody specific to native pneumolysin. Applicants respectfully traverse this ground of rejection and

direct the Examiner's attention to page 51, ln. 1 – pg. 52, ln. 20 of the instant specification where soluble mutant pneumolysin clones are selected in a screening process. For example, the five aforementioned clones were identified through this screening process and shown to possess less than 0.5% hemolytic activity when compared to wild type. As previously mentioned, the instant specification provides sufficient guidance for the skilled artisan to identify modified pneumolysins that are soluble, elicit antibodies cross-reactive with wild-type pneumolysin, and possess reduced hemolytic activity, including the single mutants, pNV103, PNV207, pNV111, and pNV211.

The Examiner further contends that the specification does not enable single amino acid substitutions at any of the recited positions besides positions 61, 148, and 195, or more than 1 amino acid substitution at more than one of the recited positions. Applicants have amended claim 35 to address the Examiner's concerns. The instant specification provides specific examples, data, and guidance for the skilled artisan to identify modified pneumolysins that are soluble, capable of eliciting antibodies which are cross-reactive with wild-type pneumolysin, hemolytically attenuated and modified to possess the characteristics as currently claimed, where the modified pneumolysin polypeptide comprises substituting at least one amino acid of SEQ ID NO:3, where the substitution is at a position selected from the group consisting of positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, and when the modified pneumolysin possesses only one substitution, the substitution is selected from the group consisting of positions 61, 148, and 195. Applicants assert that the claimed modified pneumolysin polypeptides of the invention have the claimed amino acid substitutions, as well as the functional characteristics, i.e., solubility, capability to elicit antibodies which are cross-reactive with wild-type pneumolysin, and attenuated hemolytic activity. The specification provides sufficient guidance for one skilled in the art to identify such pneumolysin mutants. In particular, the instant specification describes how to test for hemolytic activity, solubility, refoldability, and immunogenicity or antigenicity (Examples 5-7). Tables 1-5 provide examples of preferred amino acid substitutions and combinations thereof. One skilled in the art would understand that conserved amino acid substitutions at the recited positions would also be appropriate.

With respect to modified pneumolysins where amino acids are substituted at positions 61, 148, and 195 (Table 1), other amino acids having similar charge at neutral pH are also considered. For example, phenylalanine is substituted at position 195 with leucine, glycine or alanine (pg. 24, lns. 14-21). The Examiner's attention is also directed to page 28, lns. 23-28, where the mutants pNV103 and pNV207 exhibit similar immunological properties as the wild type. In order to measure hemolytic activity, the modified pneumolysin polypeptide needs to be isolated and solubilized from either the soluble fraction or inclusion bodies as described in the instant specification. Therefore, mutants such as those having the claimed amino acid substitutions and combinations are in fact soluble, elicit antibodies which are cross-reactive with wild-type pneumolysin, and possess reduced hemolytic activity as supported by the specification. Since the specification provides sufficient guidance without undue experimentation enabling the skilled artisan to test for the claimed properties, i.e., hemolytic activity, solubility, and capable of eliciting antibodies cross-reactive with wild-type pneumolysin, where the modified pneumolysin is substituted at the claimed amino acid positions, applicants respectfully request reconsideration and withdrawal of this §112, first paragraph rejection.

Further, undue experimentation is not required to identify modified pneumolysin polypeptides having the claimed properties. In fact, the amount of experimentation necessary is routine and the specification provides a reasonable amount of guidance (*PPG Indus., Inc. v. Guardian Indus. Corp.*, 75 F.3d 1558, 1564, 37 USPQ 2D 1618, 1623 (Fed. Cir. 1996)) for identifying the preferred modified pneumolysin polypeptides. The PTO has determined that the written description requirement can be met by "show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics...i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." Guidelines, 66 Fed. Reg. at 1106 (emphasis added). (*Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 296 F.3d 1316 (Fed. Cir. 2002)).

The Examiner contends that the specification lacks enablement regarding an amino acid substitution at position 243 of SEQ ID NO: 3 of claim 35. However, claim 35 as

amended allows for modified pneumolysin polypeptides, where the modified polypeptide comprises substituting at least one amino acid of SEQ ID NO:3, where the substitution is at a position selected from the group consisting of positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, and when the modified pneumolysin possesses only one substitution, the substitution is selected from the group consisting of positions 61, 148, and 195, and the modified pneumolysin polypeptide possesses the previously described characteristics. A modified pneumolysin polypeptide having a substitution at position 243 must have at least one other substitution selected from positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 255, and 257. The Examiner points to the instant specification as lacking enablement and for unpredictability. Specifically, the Examiner directs the applicants attention to Table 5B which merely shows mutants having single amino acid substitutions at positions 243, 286, and 446 and the combined substitutions at positions 243 and 446 that do not properly refold. Applicants assert the instant specification does not lack enablement nor does it show unpredictability. Claim 35 does not include a modified pneumolysin polypeptide having substituted amino acids at position 243 alone. Applicants assert that the modified pneumolysin polypeptide having an amino acid substitution at position 243 must also have at least one other substitution at positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 255, and 257. Since the mutant described in Table 5B that the Examiner bases non-enablement and unpredictability is not included in the limitations of claim 35, applicants respectfully request reconsideration and withdrawal of this §112, first paragraph rejection.

The Examiner contends that the art teaches unpredictability concerning random amino acid substitutions at positions other than those recited in claim 35. Applicants' invention provides a method that does not require undue experimentation to reliably identify and produce a soluble pneumolysin which elicits antibodies cross-reactive with wild-type pneumolysin and has attenuated hemolytic activity. The Examiner has provided no evidence indicating that practicing applicants' invention would not produce a hemolytically attenuated pneumolysin that is soluble and elicits antibodies cross-reactive with wild-type pneumolysin, nor that practicing applicants' method, as opposed to other methods described in the art, would involve undue experimentation. The Examiner simply cites

several methods in the art and alleges that they are unpredictable. However, the Examiner has not provided any reason why the alleged unpredictability of the methods known in the art has any relation to applicants' method.

Applicants remind the Examiner that claims are not necessarily invalid even if they encompass some inoperative embodiments. *Atlas Powder Co. v. E.I. Du Pont De Nemours & Co.*, 750 F.2d 1569, 1576 (Fed. Cir. 1984)("'It is not a function of the claims to specifically exclude . . . possible inoperative substances . . . .'") (quoting *In re Dinh-Nguyen*, 492 F.2d 856, 858-59 (C.C.P.A. 1974)).

> [M]any patented claims read on vast numbers of inoperative embodiments in the trivial sense that they can and do omit 'factors which must be presumed to be within the level of ordinary skill in the art,' *In re Skrivan*, 427 F.2d 801, 806 (C.C.P.A. 1970), and therefore read on embodiments in which such factors *may be* included in such a manner as to make the embodiments inoperative. There is nothing wrong with this so long as it would be obvious to one of ordinary skill in the relevant art how to include those factors in such manner as to make the embodiment operative rather than inoperative.

In *In re Angstadt*, 537 F.2d 498 (C.C.P.A. 1976), the court considered the subject matter of the application in issue, catalytic processes, unpredictable. The court also acknowledged that the scope of enablement varies inversely with the degree of unpredictability involved. The court further stated that "[a]ppellants have apparently not disclosed every catalyst which will work; they have apparently not disclosed every catalyst which will not work. *Id.* at 502. The court decided that "appellants are not required to disclose every species encompassed by their claims even in an unpredictable art such as the present record presents, each case must be determined on its own facts." *Id.* at 503. The instant invention, similar to that in *Angstadt*, provides the public with a large but finite list of variables to choose from in preparing the object of the invention. Neither of the discovered processes, *Angstadt's* and the applicants', is complicated and no special equipment or reaction conditions is required. Thus, in the instant invention, as in the case of *Angstadt*, there is no

basis for concluding that persons skilled in this art, armed with the specification and its examples, would not easily be able to determine which modified pneumolysin polypeptides are soluble, immunogenic, and have attenuated activity and which do not.

Similar to the applicants in *Angstadt*, the present applicants provide working examples in the specification of the instant invention. "If one skilled in this art wished to make and use a [soluble, attenuated pneumolysin capable of eliciting antibodies cross-reactive with wild-type pneumolysin] other than those disclosed, [he or she] would merely read [applicants'] specification for directions how to make [a soluble, attenuated pneumolysin capable of eliciting antibodies cross-reactive with wild-type pneumolysin] and could then determine whether [a soluble, attenuated pneumolysin capable of eliciting antibodies cross-reactive with wild-type pneumolysin is], in fact, formed." See, *In re Angstadt* at 503. Since applicants have provided a simple method for identifying and producing a soluble, attenuated pneumolysin capable of eliciting antibodies cross-reactive with wild-type pneumolysin, the experimentation required to determine additional soluble, attenuated pneumolysin capable of eliciting antibodies cross-reactive with wild-type pneumolysin would not be undue and definitely would not require proficiency beyond that to be expected of one of ordinary skill in the art.

Specifically, the Examiner points to Hill, et al. for support that amino acid substitutions of pneumolysin result in reduced hematolytic activity and that structure-function may be of importance. This reference supports applicants' assertion that refolding the substituted polypeptide to native protein structure is an important criteria as suggested by the instant invention. The Hill reference merely uses reduced hemolytic activity as the only factor in selecting a modified pneumolysin. Applicants assert that there is a limited number of amino acids which may be substituted in the claimed invention and that the instant specification provides sufficient guidance and enables one skilled in the art to determine without undue experimentation whether a soluble pneumolysin capable of eliciting antibodies cross-reactive with wild-type pneumolysin, where the modified pneumolysin polypeptide substitutes at least one amino acid of SEQ ID NO:3, where the substitution is at a position selected from the group consisting of positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, and when the modified pneumolysin

possesses only one substitution, the substitution is selected from the group consisting of positions 61, 148, and 195, attenuates hemolytic activity. As previously discussed, the instant specification provides enablement and guidance for the skilled artisan to identify a pneumolysin polypeptide mutant having the specific properties and claimed amino acid substitutions. The Hill reference does not address these other factors in selecting their pneumolysin mutant.

The Examiner also refers to Pizza, et al. for unpredictability in the art as to which of the site-directed substitutions at specific positions other than those claimed would eliminate toxicity while maintaining structural and functional integrity. The Pizza, et al. reference simply identifies those mutations which a) cause loss of enzymatic activity; b) do not affect enzymatic activity; or c) alter the structure. This reference also confirms that mutants having substitutions at catalytic residues and are properly assembled do not have toxicity (pg. 58, 1st col.). The art discloses that one or more substitutions in several different toxins where the toxin is structurally intact may be important for eliminating toxicity.

In particular, the Examiner contends that "it is not predictable that one or more substitution(s) with any other amino acid at the recited positions, i.e., other than valine or isoleucine substitution at position 195, the lysine substitution at position 148, and the proline substitution at position 61, would result in a modified pneumolysin that is partially soluble, haemolytically attenuated, sufficiently immunogenic or antigenic, and suitable for use as a vaccine or as a component of a conjugate vaccine" (Paper No. 37, pg. 10, ln. 28 – pg. 11, ln. 3). However, applicants respectfully point out that on page 24, lns. 14-21 of the instant specification, other amino acids having "similar charge at a neutral pH" are disclosed as suitable for substituting for wild-type amino acids. The Pizza reference that the Examiner has pointed to, specifically refers to three mutants where non-conservative amino acid substitutions were constructed. These mutants, i.e. M59, H72, and N192, having non-conservative amino acid substitutions are toxic. In particular, the M59 mutant comprises a tyrosine to methionine substitution, which is from a polar uncharged to a hydrophobic residue. The H72 mutant comprises an alanine to histidine substitution, which is from a non-polar to a positively charged basic residue. The N192 mutant comprises an arginine to asparagine substitution, which is from a positive basic to polar uncharged residue.

Applicants assert that conservative amino acid substitutions and not non-conservative substitutions are contemplated at the claimed positions. Support for such substitutions are provided in Examples 7 through 11 of the instant specification which reports that mutants having conservatively substituted amino acids are hemolytically attenuated and suitable for use as a vaccine or as a component of a conjugate vaccine while maintaining the ability to refold to a native-like structure (pg. 77, lns. 9-29). Specifically, Tables 8 and 9 and Examples 7 and 11 demonstrate that the single amino acid substituted polypeptides do have attenuated hemolytic activity and immunogenicity. Therefore, applicants assert that the instant specification provides sufficient guidance for the skilled artisan to identify and produce modified pneumolysin polypeptides that have conserved amino acid substitutions with the claimed characteristics. Furthermore, applicants assert that there is no unpredictability in the instant invention since conserved amino acid substitutions are disclosed, whereas the unpredictability in the Pizza, et al. reference is a result of non-conserved amino acid substitutions.

Referring to Pizza, et al. and Clements, et al., the Examiner contends that there is considerable unpredictability as to which of the amino acid substitution(s) at any specific position would eliminate the toxic or haemolytic effect while maintaining structural and functional competence of the protein. As previously discussed, applicants assert that the amino acid substitutions of Pizza, et al. are non-conservative, and thus the modified pneumolysin mutants remain toxic. The Examiner specifically points to the substitution of arginine at position 192 of LT with glycine which results in a less toxic, adjuvantic mutant LT (U.S. 6,019,982 to Clements, et al.) and the substitution of arginine at position 192 with asparagine which did not reduce the toxicity of LT (Pizza, et al.). Applicants once again assert that the substitutions reported in Clements, et al. and Pizza, et al. are non-conservative. Furthermore, these references simply use toxicity as the sole factor in comparing mutants; whereas, the instant invention discloses a modified pneumolysin polypeptide having several characteristics, i.e., solubility, capability to elicit antibodies which are cross-reactive with pneumolysin, and reduced hemolytic activity. The instant specification describes the limitations as claimed in claim 35 and on pages 25-26, where the amino acid substitutions for specific positions are provided. In particular, Table 4 provides specific modified

pneumolysin polypeptides having single and multiple amino acid substitutions. The specification at pages 23-28 and Tables 1-4 enable one skilled in the art to modify pneumolysin polypeptides without undue experimentation since the possible amino acids presented are limited and sufficient guidance is provided for the skilled artisan to select specific amino acids for substituting at the limited positions.

The Examiner has further pointed to one of the amino acid substitutions in claim 35, where substitution at position 243 of SEQ ID NO: 3, results in insoluble inclusion bodies. Applicants respectfully disagree with the Examiner's contention that formations of inclusion bodies is inconsistent with applicants' invention. As stated in the specification, modified pneumolysins may be derived from the soluble fraction or inclusion bodies (pg. 19, ln. 22 – pg. 20, ln. 4) from which the polypeptide must first be solubilized. Example 7 provides support and a sufficient description of the expression and purification of modified pneumolysin polypeptides derived from inclusion bodies. The Examiner contends that "substitutions that includes [sic] the position 243, resulted in insoluble (i.e., non-refoldable or not partially soluble) inclusion bodies and the attempted refolding of the mutant yielded aggregate species (see pages 57 and 58; and Table 5B" (Paper No. 37; page 11, lns. 8-10). However, the pneumolysin polypeptides having "substitutions introduced at positions 243 and 446 produced species found exclusively in the insoluble fraction as inclusion bodies" (pg. 57, ln. 28 – pg. 58, ln. 1; Emphasis added). However, the modified pneumolysin polypeptide itself is soluble even though the entity from which it is isolated, i.e. inclusion bodies, is not. One skilled in the art would understand how to solubilize the inclusion bodies both from the art and from the instant specification (pg. 59, lns. 2-28) in order to obtain the modified pneumolysin polypeptides of the claimed invention.

With respect to claims encompassing multi-substituted pneumolysins, the Examiner readily admits that Table 5A depicts pneumolysin mutants having four or five amino acid substitutions, i.e. multisubstituted pneumolysins. The Examiner further admits that "Table 4 shows that these modified pneumolysins carrying four or five specific amino acid substitutions have attenutated haemolytic activity" (Paper No. 37, pg. 14, lns. 10-12). However, the Examiner contends that "there is no showing that these modified pneumolysins are 'partially-soluble' and possess the capacity to bind with an antibody to native

pneumolysin, i.e., retain pneumolysin-specificity or pneumolysin-neutralizing ability" (Paper No. 37, pg. 14, lns. 12-14). Applicants respectfully direct the Examiner's attention to Example 5 of the instant specification (pgs. 49-57) which reports the process of selecting modified pneumolysin expressing modified pneumolysin without toxic effects. The screening method for identifying colonies that expresses modified pneumolysin polypeptides is based on the following characteristics: 1) no hemolytic activity, 2) substantially full-length, 3) partially soluble, and 4) monomeric and refoldable when isolated from inclusion bodies. Five clones were selected based on this method, i.e., pNVJ1, pNVJ20, pNVJ22, pNVJ45, and pNVJ56 which have multiple amino acid substitutions. The screening process is based on identifying modified pneumolysin polypeptides, such as those reported in Tables 1, 2, and 3, provided they retain at least one epitope recognized by an antibody which binds to the mature pneumolysin (pgs. 25-26). Furthermore, on page 34, ln.12 – page 35, ln. 5 of the instant specification, applicants point out that modified pneumolysins may be altered at one or more residues sites "provided they allow for refolding of the pneumolysin." These clones have the characteristics of hemolytic activity, substantially full-length, soluble, and monomeric/refoldable, but are also capable of retaining pneumolysin specificity. Since the multi-substituted modified pneumolysin polypeptides that are soluble, capable of eliciting antibodies which are cross-reactive with wild-type pneumolysin, and have reduced hemolytic activity are identified by the screening method disclosed in the instant specification, sufficient guidance is provided throughout the specification for one skilled in the art to identify and produce such a modified pneumolysin. Therefore, applicants respectfully request reconsideration and withdrawal of this §112, first paragraph rejection.

The Examiner further contends that "the art reflects that substitutions of critical residues at specific positions in an amino acid sequence could result in a peptide which may induce an antibody that may not recognize or bind to the native polypeptide" (Paper No. 37, pg. 16, lns. 9-11). The Examiner further mentions McGuinnes, et al. (1991; 1993) as reporting that a single amino acid change in an epitope can result in structural changes and Houghten, et al. (1986) reports the importance of one or more amino acid residues and their positions in peptide antigen-antibody interactions. However, applicants assert that amino acid substitutions are at positions that do not affect the epitopes which elicit

antibodies that bind the native toxin molecule (pg. 7, lns. 11-16). Applicants respectfully direct the Examiner's attention to Example 7, specifically Table 9, of the instant specification which shows that there is significant binding of wild type pneumolysin to antibodies elicited by modified pneumolysin polypeptides, specifically pNV211, pNV111, pNV103, and pNV207. Examples 7 and 9 also provide guidance as to immunizing rabbits and testing for the presence of antibodies against wild type pneumolysin. Moreover, one of the characteristics of the modified pneumolysin polypeptides of the instant invention is refoldability. The instant invention describes and enables modified pneumolysins possessing the claimed elements of solubility, capability to elicit antibodies which are cross-reactive with wild-type pneumolysin, and reduced hemolytic activity, as described in Example 5. The modified pneumolysin polypeptides are selected using the previously discussed criteria, including the ability to bind antibodies directed to native pneumolysins. Therefore, since the selected clones maintain attenuated hemolytic activity and their ability to bind antibodies directed against native pneumolysins as well as solubility, modified pneumolysin polypeptides having one or more amino acid substitutions are described and enabled in the present specification. Further, the instant specification provides sufficient guidance for one skilled in the art to test for and identify such modified pneumolysin polypeptides (i.e., CD and fluorescence spectroscopy for refoldability, ELISA for immunogenicity, hemolytic activity assay).

The Examiner also contends that the applicants did not have possession of any of the modified pneumolysins having the required functions as covered by claims 65-79. Claims 65- 68 are directed to mutants having specific amino acids substituted at positions 61, 148, 195, and 243. Claims 69-79 are directed to mutants having specific combination of substitutions and their specific amino acids. The instant specification reports that substitutions for amino acids at positions 61, 148, and 195 are not limited to the preferred amino acids listed in Table 1, but also include "those having similar charge at neutral pH" (pg. 24, lns. 14-21). Additionally, the instant specification enables one skilled in the art to modify pneumolysin at the preferred positions, such that the modified pneumolysins are hemolytically attenuated, soluble and refoldable having sufficient immunogenic and antigenic activity. Furthermore, mutants pNVJ1, pNVJ45, pNVJ20, pNVJ22 as described in Tables 4

and 5A are directed to the combination of substitutions of claims 69-79. Therefore, applicants assert they had possession of the mutants of claims 65-79.

Lazar, et al. is provided as a reference by the Examiner for demonstrating that a substitution of Leu with a conservative amino acid residue (Ile or His) of TGF alpha alters biological activity. The Examiner also points to Hansen et al. (WO 98/06851) which reports substitution of a hydrophilic aspartic acid for another hydrophilic asparagine at position 3 of a particular peptide resulted in abolishing reactivity of the peptide with the bactericidal 10F3 monoclonal. However, applicants respectfully bring to the Examiner's attention that the substitutions are not equivalent. Histidine is an uncharged or positively charged hydrophilic amino acid, while leucine is a hydrophobic amino acid. Aspartic acid carries a negative charge while asparagine is uncharged. Applicants do note that the substitution of leucine with isoleucine is a conservative substitution; however, the Examiner is attempting to mix and match different combinations of characteristics to refute the possibility of substituting amino acids which result in similar functions. Applicants assert that modified pneumolysin polypeptides having conservative amino acid substitutions that are soluble, capable of eliciting antibodies which are cross-reactive with wild-type pneumolysin, and have reduced hemolytic activity are provided. The claimed mutants do not possess one or two of these characteristics, rather they have all of the claimed elements. The Lazar, et al. and Hansen, et al. references simply refer to one characteristic and not the entire invention as claimed. Since the specification describes how one skilled in the art would be able to identify the claimed modified pneumolysin polypeptides by testing for hemolytic activity, immunogenicity, and solubility, there is sufficient guidance and enablement for the skilled artisan to identify modified pneumolysin polypeptides as claimed. Thus, applicants respectfully request reconsideration and withdrawal of this §112, first paragraph rejection.

35 U.S.C. §112, Second Paragraph

25) Claims 35-37, 42, 53 and 60-79 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Applicants respectfully traverse the Examiner's rejection.

(a) The Examiner contends that claim 35 is rejected to for the vague, indefinite, and confusing recitation "amino acid substitution selected from the group consisting of residues." Applicants respectfully traverse this ground of rejection, but in order to expedite prosecution of this application, claim 35 has been amended by replacing the term "residues" with - -substitutions at position- - to address the Examiner's concerns. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

(b) The term "partial" in claim 35 has been rejected as being a relative term which renders the claim indefinite. Applicants respectfully traverse the Examiner's grounds of rejection. However, in order to expedite prosecution of this application, claim 35 has been amended to delete the phrase "partial." Applicants respectfully request reconsideration and withdrawal of this §112, second paragraph rejection.

(c) Claim 53 has been rejected for allegedly being vague and indefinite in the recitation "polypeptide according to claim 35 wherein the polypeptide is obtained by randomly mutating." Applicants respectfully traverse this rejection; however, in order to expedite prosecution of this application, claim 53 has been amended by removing its dependency to claim 35, thereby amending claim 53 into an independent claim, in order to address the Examiner's concerns. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

(d) The Examiner has rejected claim 62 for not having antecedent basis for the recitation "The" vaccine. Applicants have amended this claim by replacing "The" with - - A- - in order to expedite prosecution. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

(e) Claim 64 is rejected to for being unclear as to the antecedent basis for the recitation "the polysaccharide is a bacterial polysaccharide." Applicants have amended the claim to delete the phrase, "polysaccharide is a" to read - -the bacterial polysaccharide is from a bacterium- - in order to address the Examiner's concerns. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

(f) The Examiner has rejected claim 65 for lacking antecedent basis for the recitation "at least one amino acid substitution." Claim 65 has been amended by

replacing the phrase "at least" with - -said modified pneumolysin polypeptide having only- - in order to put the claim in proper format as suggested by the Examiner. Applicants respectfully request reconsideration and withdrawal of this §112, second paragraph rejection.

(g) Claim 65 has been rejected as allegedly being vague and indefinite in the recitation of "amino acid substitution is a proline or hydroxyproline at residue 61." Applicants respectfully traverse this rejection. However, claim 65 has been amended to read, - -amino acid substitution is a proline or hydroxyproline substitution at position 61- - as suggested by the Examiner. Applicants respectfully request reconsideration and withdrawal of this §112, second paragraph rejection.

(h) Claim 66 has been rejected for allegedly lacking antecedent basis for the recitation "at least one amino acid substitution." Applicants have amended claim 66 by putting the claim in proper format, specifically, by replacing the phrase "at least" with - -said modified pneumolysin polypeptide having only- - in order to address the Examiner's concerns. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

(i) The Examiner has rejected claim 66 for being indefinite in the recitation "amino acid substitution is a lysine, arginine or histidine at residue 148." Applicants respectfully traverse this ground of rejection, but have amended the claim by replacing "histidine at residue 148" with - -histidine substitution at position 148- - in order to address the Examiner's concerns. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

(j) Claims 67 and 68 have been rejected for being indefinite in the recitation "amino acid substitution is a....at residue...." These claims have been amended by replacing "at residue" - -substitution at position- - in order to address the Examiner's concerns. Applicants respectfully request reconsideration and withdrawal of this §112, second paragraph rejection.

(k) With respect to claims 65-69, 71-73, 75, 77, and 78, the Examiner has suggested that applicants replace the recitation "residue" with - - position- - and "residues" with - - positions- -. In order to expedite prosecution of this application, claims 65-69, 71-73, 75, 77, and 78 have been amended as suggested by the Examiner in order to address the

Examiner's concerns. Reconsideration and withdrawal of this §112, second paragraph is respectfully requested.

(l) Claims 70, 72, 74, 76, and 79 have been rejected for allegedly not distinctly claiming the subject matter with respect to the recitation "for residue." The Examiner has suggested that applicants replace the recitation "for residue" with - - at position - -. Applicants respectfully traverse the Examiner's rejection, but in order to expedite prosecution of this application, claims 70, 72, 74, 76, and 79 have been amended as suggested by the Examiner. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

(m) The Examiner has rejected claims 36, 37, 42, 53, and 60-79 for being indefinite because of the alleged vagueness or indefiniteness identified in the base claim 35. Applicants have amended base claim 35 and thus, depending claims 36, 37, 42, 53, and 60-79 are not indefinite. Reconsideration and withdrawal of this §112, second paragraph rejection is respectfully requested.

Objection(s)

26) Claim 73 has been objected to for lacking a period at the end of the claim. Applicants have amended the claim in order to address the Examiner's concerns. Reconsideration and withdrawal of this objection is respectfully requested.

CONCLUSION

As required by 37 C.F.R. §1.121, "marked up" versions of the amended claims and of the replacement paragraphs of the specification are attached herewith with additions indicated by underlining and deletions by brackets.

Applicants respectfully submit that the instant application is in condition for allowance. Entry of the amendment and an action passing this case to issue is therefore respectfully requested. In the event that a telephone conference would facilitate examination of this application in any way, the Examiner is invited to contact the undersigned at the number provided.

Allowance of the pending claims is respectfully requested. Early and favorable action by the Examiner is earnestly solicited.

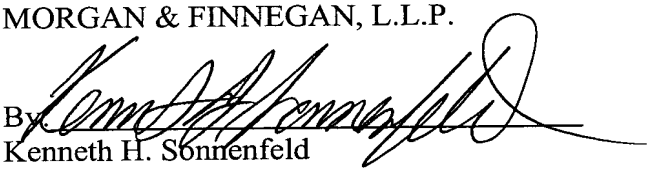
AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for the timely consideration of this amendment under 37 C.F.R. §§ 1.16 and 1.17, or credit any overpayment to Deposit Account No. 13-4500, Order No. 3842-4036US2.

In the event that an extension of time is required, or which may be required in addition to that requested in a petition and for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. 13-4500, Order No. 3842-4036US2. A DUPLICATE COPY OF THIS SHEET IS ATTACHED.

Respectfully submitted,
MORGAN & FINNEGAN, L.L.P.

Dated: February 14, 2003

By: 
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

35. (twice amended) A modified pneumolysin polypeptide, [wherein the polypeptide is partially soluble has attenuated hemolytic activity, and] wherein the modification of the polypeptide comprises substituting at least one an amino acid of[sequence having] SEQ ID NO:3, wherein said substitution is at a position [modified to possess at least one amino acid substitution selected from the group consisting of residues] selected from the group consisting of positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, wherein when said modified pneumolysin possesses only one substitution, said one substitution is selected from the group consisting of positions 61, 148, and 195, and wherein said modified pneumolysin is soluble, elicits antibodies which are cross-reactive with wild-type pneumolysin, and has attenuated hemolytic activity.

53. (twice amended) [The polypeptide according to claim 35] A modified pneumolysin polypeptide, wherein the polypeptide is soluble, elicits antibodies which are cross-reactive with wild-type pneumolysin, has attenuated hemolytic activity, and wherein the modification of the polypeptide is obtained by randomly mutating a nucleic acid molecule encoding a pneumolysin polypeptide.

62. (twice amended) [The] A vaccine comprising the polypeptide according to claim 35 and a pharmaceutically acceptable carrier.

64. (twice amended) The vaccine according to claim 63, wherein the [polysaccharide is a]bacterial polysaccharide [and]is from a bacterium selected from the group consisting of *Haemophilus influenzae* type b; meningococcus group A, B, or C; group A streptococcus or group B streptococcus type Ia, Ib, II, III, V, or VIII; and one or more of serotypes 1-23 of *S. pneumoniae*.

65. (amended) The polypeptide according to claim 35, wherein said [at least] modified pneumolysin polypeptide having only one amino acid substitution is a proline or hydroxyproline substitution at [residue]position 61.

66. (amended) The polypeptide according to claim 35, wherein said [at least] modified pneumolysin polypeptide having only one amino acid substitution is a lysine, arginine or histidine substitution at [residue]position 148.

67. (amended) The polypeptide according to claim 35, wherein said [at least] modified pneumolysin polypeptide having only one amino acid substitution is a leucine, glycine, alanine, valine or isoleucine substitution at [residue]position 195.

68. (amended) The polypeptide according to claim 35, wherein said [at least one] amino acid substitution is an arginine, valine, glutamic acid, or serine substitution at [residue]position 243.

69. (amended) The polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at [residues]positions 17, 18, 61, 66 and 101.

70. (amended) The polypeptide according to claim 69, wherein the substitutions consist of arginine [for residue]at position 17, asparagine [for residue]at position 18, proline [for residue]at position 61, tyrosine [for residue]at position 66, and threonine [for residue]at position 101.

71. (amended) The polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at [residues]positions 41, 172, 195 and 255.

72. (amended) The polypeptide according to claim 71, wherein the substitutions consist of glycine [for residue]at position 41, alanine [for residue]at position 172, isoleucine [for residue]at position 195, and glycine [for residue]at position 255.

73. (amended) The polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at [residues]positions 63, 127, 128 and 148.

74. (amended) The polypeptide according to claim 73, wherein the substitutions consist of serine [for residue]at position 63, glutamic acid [for residue]at position 127, histidine [for residue]at position 128, and lysine [for residue]at position 148.

75. (amended) The polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at [residues]positions 33, 46, 83, 239 and 257.

76. (amended) The polypeptide according to claim 75, wherein the substitutions consist of threonine [for residue]at position 33, threonine [for residue]at position 46, serine [for residue]at position 83, arginine [for residue]at position 239 and glycine [for residue]at position 257.

77. (amended) The polypeptide according to claim 75, wherein the substitutions at [residues]positions 33, 46 and 83 are [either] selected from the group consisting of a serine, threonine, asparagine, glutamine, tyrosine [or] and cysteine; the substitutions at [residue]position 239 is [either] selected from the group consisting of a lysine, arginine or histidine; and the substitution at [residue]position 257 is [either] selected from the group consisting of a leucine, glycine, alanine, isoleucine [or] and valine.

78. (amended) The polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at [residues]positions 45, 102, 189 and 195.

79. (amended) The polypeptide according to claim 78, wherein the substitutions consist of alanine [for residue]at position 45, glycine [for residue]at position 102, arginine [for residue]at position 189, and valine [for residue]at position 195.

Please replace the specification as follows:

IN THE SPECIFICATION

Please replace the paragraph on page 44, ln. 18 through page 45, ln. 5 with the following:

Bacterial Strains and Plasmids. *Streptococcus pneumoniae* serotype 14 (ATCC, [Rockville, MD]10801 University Boulevard, Manassas, VA 20110-2209) was used in this study for isolation of genomic DNA. *E. coli* strain DH5 (Life Technologies, Gaithersburg, MD) was used for initial cloning and production of plasmid DNA. *E. coli* strain BL21 (DE3)ompA, used for protein expression, was derived from BL21 (BE3) (Novagen) (see U.S. Patent

No. 5,439,808 for details). *S. pneumoniae* was grown overnight in Todd-Hewitt (TH) broth at 37C without shaking under 7.5% CO_2. *E. coli* strains were grown in Luria-Bertani (LB) broth, supplemented with carbenicillin (50-100 μg/ml) or kanamycin (50 μg/ml) as needed. The plasmid vectors pUC-19 and/or pBluescript II SK+ (Stratagene) were used for cloning fragments to be sequenced and the plasmids pET-17b and pET-24a (Novagen) were used for cloning fragments to be expressed.

Please replace the paragraph on page 51, ln. 23 through page 52, ln. 20 with the following:

Clones containing soluble pneumolysin are selected for the next step in the screening procedure, which consists of discarding the supernatant by aspiration, washing the pellet with TEN buffer twice, and solubilizing the pellet in 5 ml of 8 M urea prepared in TEN buffer. After sonicating for 2 min, the urea solution is quickly centrifuged to remove aggregates and added dropwise to 45 ml of refolding solution, under constant stirring at 4 °C. The refolding solution is then loaded onto a 2 ml DEAE-[Sepharose] SEPHAROSE-FF column, pre-equilibrated in Buffer A (25 mM Tris.HCl, pH 8.0). The column is washed with Buffer A and the bound protein is eluted with a gradient of 0 to 1 M NaCl. The properly refolded pneumolysin mutant should elute as a single peak between 13 and 20% Buffer B (25 mM Tris.HCl, 1 M NaCl, pH 8.0) similarly to what is observed for the wild-type. The protein peak is further analyzed by HPLC on a [Superose] SUPEROSE 12 column and both elution time, aggregate/monomer ratio, and hemolytic activity are evaluated (see Table 4). The selected mutant(s) should present a single monomeric species with a Stokes radius comparable to the wild-type. Five clones (pNVJ1, pNVJ20, pNVJ22, pNVJ45, pNVJ56) with high yields of monomeric modified polypeptides were selected for further analysis including nucleic acid sequencing. The amino and nucleic acid substitutions of these

clones are shown in Tables 5A and 6. Throughout the specification and claims, proteins are given the name of the vector that encodes them.

Please replace the paragraph on page 59, lns. 2-28 with the following:

Pneumolysin expressed in *E. coli* cells harboring the expression vector pNV19 was isolated from inclusion bodies by resuspending and lysing the cells in TEN buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA pH 8.0), with an air driven cell disrupter (Stansted Fluid Power Ltd.) under a pressure of 8,000 psi. The cell lysate was centrifuged at 13,000 rpm at 4C for 20 minutes; both pellet and supernatant were saved for isolation of soluble and aggregated pneumolysin, respectively. The inclusion bodies were washed three times with TEN buffer and stored at -70C Purification and subsequent refolding were achieved by solubilizing the inclusion bodies in an 8 M urea solution (freshly prepared in TEN buffer), followed by PEG-assisted refolding. Polypeptide solutions in 8 M urea (200 μg/ml) were diluted 10-fold by drop-wise addition to a refolding solution, consisting of 20 μM of PEG 8,000 in 25 mM Tris-HCl, pH 8.0, under constant stirring at 4C. The sample was clarified and loaded into a DEAE-[Sepharose] SEPHAROSE Fast Flow ion exchange column (Pharmacia) equilibrated in 25mM Tris-HCl, pH 8.0. A gradient of 0-1 M NaCl was applied and pneumolysin containing fractions were identified by detection of hemolytic activity, as described below, and by SDS-PAGE. The purified fractions were concentrated by using an Amicon concentrator and PM30 membrane. Aliquots of purified polypeptide were tested for hemolytic activity, and analyzed by SDS-PAGE and size exclusion chromatography, using a [Superose] SUPEROSE 12 column.

Please replace the paragraph on page 63, lns. 5-24 with the following:

PnC type 14 polysaccharide (ATCC Lot #2016107) (390 mg) was dissolved in 16 ml of 0.5 N NaOH, and the solution was heated at 70C for 3 hours. Following cooling of the solution, 1.93 ml of glacial acetic acid was

added to bring the pH to 4. After addition of 3 ml of 5% (w/v) $NaNO_2$, the reaction mixture was kept stirring at 4C for 2 hours. The sample was then diluted to 50 ml with deionized water and the pH was adjusted to 7 with 0.5 N NaOH. Excess reagents were dialyzed out by diafiltration with DI water through a [Spectra/Por molecularporous] SPECTRA/POR molecular porous membrane tubing (MWCOL:3,500), and the retentates freeze-dried. The deaminated type 14 polysaccharide was then molecular sieved on a [Superdex] SUPERDEX G-200 (Pharmacia) column using PBS as eluent. Fractions eluting from the column with molecular weight between 5000 and 15,000 as determined by Chromatography/Multiangle Laser Light Scattering using a [Superose] SUPEROSE 12 column (Pharmacia) were pooled and dialyzed against DI water through a [Spectra/Por molecularporous] SPECTRA/POR molecular porous membrane tubing (MWCOL 3,500) and freeze-dried.

Please replace the paragraphs on page 64, ln. 3 through page 65, ln.3 with the following:

Modified pneumolysin polypeptides in 0.2 M phosphate buffer (pH 8) at a concentration of 5 mg/ml were mixed with 2.5 equivalents (by weight) of PnC 14 polysaccharide-fragment together with 2 equivalents (by weight) of recrystallized sodium cyanoborohydride . Reaction mixtures were incubated at 37°C for 24 hours. Conjugates were then purified from the free components by passage through a [Superdex] SUPERDEX G200 (Pharmacia) column using PBS containing 0.01% thimerosal as an eluent. Fractions eluting from the column were monitored on a Waters R403 differential refractometer and by UV spectroscopy at 280 nm. The fractions containing the conjugates were pooled, sterile-filtered through a 0.22 μm Millipore membrane and then stored at 4C. Polypeptide and carbohydrate content were measured by the methods of Bradford and Dubois respectively. Polysaccharide content in the resulting conjugates were approximately 30%.

Tetanus toxoid conjugates for use as control, were also produced as described above and as follows: Tetanus toxoid (Serum Statens Institute) was first passed through a molecular sieve column ([Superdex] SUPERDEX G-200 Pharmacia) in order to obtain the monomer form of the toxoid. For conjugation, 12 mg of the monomer and 36 mg of the PnC 14 polysaccharide-fragments were dissolved in 600 µl of 0.2 M phosphate buffer pH 7.2. Recrystallized sodium cyanoborohydride (24 mg) was then added to the solution which was then incubated at 37°C for 3-days. The conjugate was purified as above. The conjugates had polysaccharide contents in the 25-30% range (see Table 10).

Please replace the paragraphs on page 66, lns. 4-28 with the following:

Micro titer plates (Nunc Polysorb ELISA plates) were sensitized by adding 100 µl of type 14 polysaccharide-fragment (MW ca: 10,000)/HSA conjugate (2.5 µg/ml) in PBS. The plates were sealed and incubated at 37C for 1 hour. The plates were washed with PBS containing 0.05% [Tween 20] TWEEN 20 (PBS-T) and blocked with 0.5% (w/v) BSA in PBS for 1 hour at room temperature. The wells were then filled with 100 µl of serial two-fold dilutions in PBS-T plates, 100 µl of peroxidase labeled goat anti-mouse IgG (H+L) (Kirkegaard and Perry Laboratories), and then washed five times with PBS-T. Finally, 50 µl of TMB peroxidase substrate (Kirkegaard and Perry Laboratories) were added to each well, and following incubation of the plates for 10 minutes at room temperature, the reaction was stopped by the addition of 50 ul of 1 M H_3PO_4. The plates were read at 450 nm with a Molecular Device Amex microplate reader using 650 nm as a reference wavelength.

Inhibition ELISA assay.

Microtiter plates (NUNC Polysorp) were coated with PLY (20 ng in 100 mL to each well) in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) for one hour at 37°C. After washing the plates with PBS + 0.05%

[Tween 20] TWEEN 20 (PBST), the plates were post-coated with 150 mL of PBS + 0.1% BSA, rewashed, and stored at 4°C until used.

Please replace the paragraph on page 67, lns. 1-21 with the following:

Hyperimmune rabbit anti-PLY was diluted in PBST, added to the PLY coated plates, and incubated at room temperature for 1 h. After washing, 100 mL of goat anti-rabbit Ig-HRP conjugate (KPL) diluted in PBST[ween] according to the manufacturer's instructions were added to each well. The plate was incubated at room temperature for one hour and then washed again. 100mL of TMB microwell substrate (KPL) were added to each well. The reaction was stopped after 10 minutes by the addition of TMB one-component stop solution (KPL) and the $OD_{450\ nm}$ was immediately read. The dilution corresponding to 1/2 the maximum signal was chosen for the inhibition study. PLYD mutants as well as PLY as a control were diluted serially in three-fold ingrements in PBST containing the rabbit antiserum diluted such that the final mixture contained the dilution which gave half-maximal activity and applied immediately to the coated microtiter plates in duplicate. The plates were incubated at room temperature for one hour and processed. Inhibition was determined as percent of maximum signal achieved with dilute antiserum in the absence of any inhibitor.

Please replace the paragraph on page 71, lns. 6-24 with the following:

The oxidized PSs were separately coupled to recombinant pneumolysoid mutant 207 in which amino acid Phe residue 195 was replaced by Ile. In brief, the oxidized PSs and the protein (5 mg/ml) in 0.2 M sodium phosphate buffer were combined at a PS/protein ratio of about 2.5:1 by weight at room temperature and sodium cyanoborohydride (2 equivalents by weight) was then added. The conjugation mixtures were incubated at 37C for 2 days. After reduction of the residual aldehydes of the conjugated PS, with excess

$NaBH_4$, the conjugates were purified from the reaction mixtures by passage through a column of [Superdex] SUPERDEX 200 PG (Pharmacia) eluted with PBS containing 0.01% thimerosal as the preservative, except for the type 23 conjugate where the conjugate was loaded onto a Q [Sepharose] SEPHAROSE Fast Flow column, and eluted with 10 mM Tris-HCl, pH 7.5 using a gradient of 0.5 M NaCl. Fractions corresponding to the conjugates were pooled and analyzed for protein and carbohydrate content as described in example 8 (see Table 12).